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Chemiluminescence study on the peroxidation of linoleic acid initiated by the reaction of ferrous iron with hydrogen peroxide

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Linoleic acid was used as a model system to study lipid peroxidation initiated by the reaction of ferrous iron with hydrogen peroxide. Low-level chemiluminescence of the peroxidation was measured with a high-sensitivity single-photon counter. It was found that the luminescence primarily comes from the dimol reaction of singlet oxygen and that the peak intensity of emission is a quadratic function of the concentration of either Fe^{2+} or H_2O_2, provided that the other Fenton reagent is in great excess. Under the same conditions, analysis on reaction kinetics shows a linear relationship between the maximal level of the initiator formed by the Fenton reaction and the initial concentration of Fe^{2+} or H_2O_2. This implies that the peak intensity of the chemiluminescence may be a good index of the maximal level of the initiator.

1. Introduction

The peroxidation of lipid initiated by the reaction of Fe^{2+} with H_2O_2 has been studied by several groups in order to understand the nature of the initiator, especially the involvement of ferric ion [1,2]. In those investigations, peroxidation was mainly monitored by measuring the products of peroxidation, such as malondialdehyde (MDA) and the conjugated dienes, which are only indexes of overall peroxidation reactions. One should bear in mind that such measurements are not directly related with the initial rate of peroxidation and the initial level of the initiator. Therefore, it is desirable to have an approach by which one can observe the initial peroxidation rate and bridge it with effectiveness of the initiator.

Methodologically, a number of techniques are available for measuring the rate of peroxidation of membrane lipid or fatty acids. Perhaps the simplest method of measuring the overall rate of peroxidation in vitro is to determine oxygen uptake in a O_2 electrode. However, this method can hardly be used in vivo, since the rate of O_2 uptake caused by radical reactions may be only a small percentage of total cellular O_2 uptake [3]. Another method is to measure the loss of unsaturated fatty acids after extracting and hydrolysing membrane lipids. But further oxidation of the fatty acids may occur during the handling of the extracts. The third class of methods is to measure the products of lipid peroxidation, among which the most frequently used is to measure the MDA by the thiobarbituric acid (TBA) test [4]. Except for the measurement of O_2 uptake, all other methods cannot directly measure the actual rate of peroxidation.

Chemiluminescence seems to be an overall index of radical reactions taking place in lipid peroxidation. It is a noninvasive technique for mea-

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suring 'oxidative stress' in intact tissues, and is potentially able to measure the rate of peroxidation [5,6]. However, how the chemiluminescence reflects the level of the initiator which initiates lipid peroxidation and the radical reactions which generate excited states have not yet been well established. In this paper, we report a quadratic dependence of the chemiluminescence on the concentration of the initiator in lipid peroxidation and show the potential of chemiluminescence for quantitative monitoring of the initial rate of peroxidation.

2. Material and methods

2.1. Materials

Linoleic acid (LA) was purchased from Fluka (Switzerland), pure iron wires from Chinoin (Budapest), hydrogen peroxide (A.R. ~ 30 wt% in water) from Beijing Chemical Co. and dimethyl sulfoxide (DMSO) from J.T. Baker Chemical Co.

2.2. Preparation of solutions

0.3 mM linoleic acid was solved in 0.9% NaCl (pH 6.5) containing 0.7 μM DMSO as solubilizer. 10 mM $FeCl_2$ solution (pH 3) was freshly prepared by dissolving 0.1396 g pure iron wire in 5 ml of 3.5 M HCl at 40°C overnight, followed by dilution to 250 ml with double-distilled water and pH adjustment with NaOH.

2.3. Chemiluminescence detection

2 ml of 0.3 mM LA was mixed with different volumes of 10 mM $FeCl_2$ in a quartz cuvette to make linoleic acid solutions containing various concentrations of Fe^{2+}. 0.9 ml of H_2O_2 (concentration depending on the particular experiment) was injected into the above LA/Fe^{2+} solution to initiate peroxidation. The low-level chemiluminescence caused by lipid peroxidation was detected with a laboratory-made computerized high-sensitivity single-photon counter (SPC). In order to achieve high sensitivity, the EM-9558B photomultiplier in the SPC was cooled to −17°C using cold circulating alcohol.

In the SPC, the sample compartment can be kept at any desired temperature between 25 and 50°C. A series of interference filters can be mounted in front of photomultiplier for measurement of the luminescence spectrum. The spectral response of the counter associated with filters was determined by a self-calibrated silicon photodiode with respect to a tunable monochromatic light source consisting of a tungsten lamp (Philips) and a grating monochromator (WDS-3 type) according to the method developed by Geist and Zalewski [7].

2.4. Estimation of H_2O_2

The actual concentration of H_2O_2 used for injection in initiating the peroxidation of LA/Fe^{2+} was estimated by the iodide-molybdate method [8]. In the assay, the reagent must be freshly prepared by equal-volume mixing of solution A (66 g KI + 2 g NaOH + 0.2 g $(NH_4)_2MoO_4 \cdot 4H_2O$ l^{-1}) and solution B (20 g $KHC_8H_4O_4$ l^{-1}). The test solution was mixed with an equal volume of the reagent, and the spectrophotometric estimation of the I_3^- formed ($\epsilon = 2.54 \times 10^4$ M^{-1} cm^{-1} at 350 nm) gives the concentration of H_2O_2.

3. Results

3.1. Profile of the chemiluminescence

The profile of the light emission from the peroxidation of linoleic acid is shown in fig. 1. The luminescence rises steeply and reaches its maximum about 5 s after injection of H_2O_2, subsequently decaying and returning to an undetectable level within about 3 min. The rising part of the profile corresponds to the phase of initiation, and the decay to the phase of termination. No luminescence can be detected if H_2O_2 is injected into the LA solution containing either no Fe^{2+} or only Fe^{3+}.

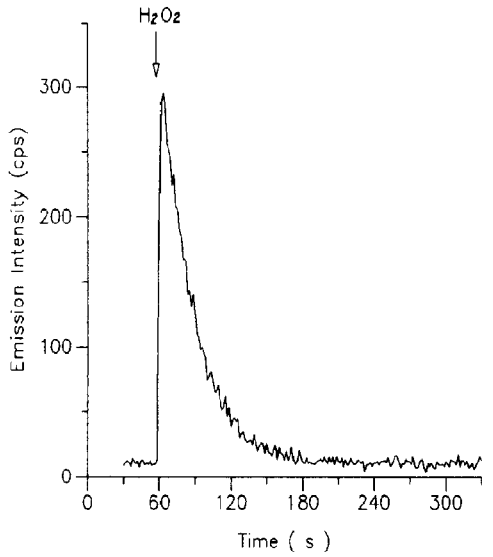

Fig. 1. Profile of chemiluminescence initiated by injection of 0.9 ml of 3.3 mM H_2O_2 into 2.1 ml of 0.3 mM linoleic acid in 0.9% NaCl solution containing 0.14 mM $FeCl_2$ (pH 5.0) at 35°C. Luminescence was measured with a high-sensitivity single-photon counter.

3.2. Dependence of chemiluminescence on the initial concentration of Fe^{2+} when H_2O_2 is in considerable excess

For simplicity of the reaction kinetics, the chemiluminescence was measured with 0.2 mM LA solution containing various concentrations of Fe^{2+} (from 0.03 to 0.12 mM) after adding excess H_2O_2 (0.5 mM). All data are the means of three independent measurements, and the standard deviations are calculated on the bases of statistical fluctuation of photoelectron counts and variance of the three independent measurements. The results are shown in fig. 2, in which the square root of the peak intensities is plotted vs the initial concentration of Fe^{2+}. It is clearly shown that a linear regression can fit the experimental data very well.

3.3. Dependence of chemiluminescence on the initial concentration of H_2O_2 when Fe^{2+} is in considerable excess

Similarly, the chemiluminescence bursts from 0.2 mM LA solution containing 1 mM Fe^{2+} initiated by injection of different concentrations of H_2O_2 were recorded. The data are shown in fig. 3. Again, a good linearity exists between the square root of the peak intensities and the initial concentration of H_2O_2.

3.4. Dependence of chemiluminescence on the initial concentration of Fe^{2+} and H_2O_2 when both have equal values

The chemiluminescence bursts from 0.2 mM LA solution initiated by the reaction of Fe^{2+} with H_2O_2 of equal concentration were recorded. The results are shown in fig. 4. The curve calculated by computer shows that the data can be well fitted with the following equation:

$$I = \frac{a \cdot [c]^4}{(1 + b \cdot [c])^4}$$

where constants a and b are equal to 3.39×10^{17} M^{-4} cps and 1.87×10^3 M^{-1}, respectively.

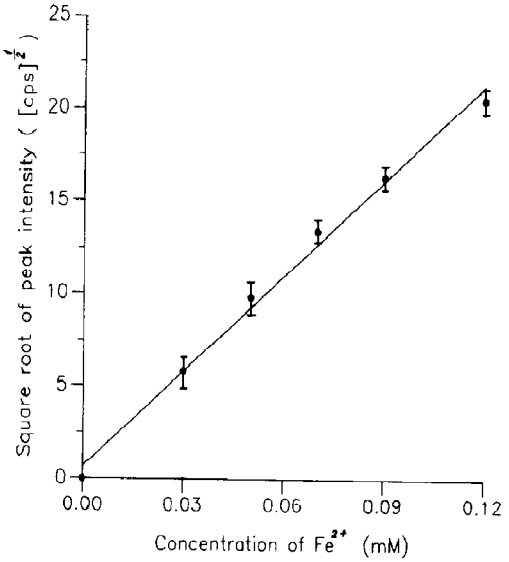

Fig. 2. Dependence of peak intensity of the chemiluminescence on the initial concentration of Fe^{2+}. Lipid peroxidation in 0.2 mM linoleic acid of 0.6% NaCl solution was initiated by reaction of various concentrations of Fe^{2+} with 0.5 mM H_2O_2 at 35°C. Ordinate: square root of the peak intensity; bars indicate the standard deviation of the data. The straight line represents the linear regression of the data.

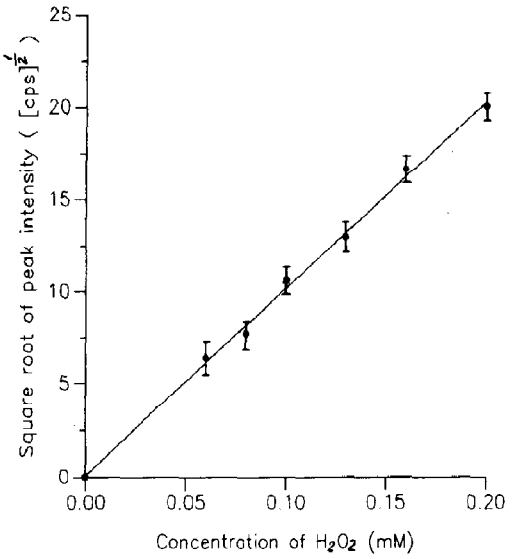

Fig. 3. Dependence of peak intensity of the chemiluminescence on the initial concentration of H_2O_2. Lipid peroxidation in 0.2 mM linoleic acid of 0.6% NaCl solution was initiated by reaction of various concentrations of H_2O_2 with 1 mM Fe^{2+} at 35° C. Plotting procedure same as that in fig. 4.

3.5. *Emission spectrum of the chemiluminescence*

The emission spectrum of the peroxidation of 0.2 mM linoleic acid initiated by the reaction of 0.2 mM Fe^{2+} with 0.5 mM H_2O_2 was determined by recording the chemiluminescence bursts with different interference filters, followed by correcting for the spectral response of the photomultiplier associated with filters. The spectrum is shown in fig. 5; three maxima occur at 488.4, 572.8 and 639.2 nm, the wavelengths of the available filters. These emission bands are quite well in accord with the wavelengths of the photons produced by the dimol reaction of singlet oxygen (see table 1) [9].

4. Theoretical treatment

Commonly, the hydroxyl radical, $^{\cdot}OH$, has been considered as the initiator of lipid peroxidation in biological systems where Fe^{2+} and H_2O_2 are present [10]. The well-known Fenton reaction (reaction 1) has been proposed for the generation of the $^{\cdot}OH$ radical.

$$Fe^{2+} + H_2O_2 \xrightarrow{k_1} Fe^{3+} + {}^{\cdot}OH + OH^- \qquad (1)$$

However, some investigators proposed that an iron-bound oxygen species instead of the $^{\cdot}OH$ radical may be the product of reaction 1 and be responsible for initiating lipid peroxidation in biological systems [11]. One frequently proposed form of such a species is the ferryl ion which may be formed by the reaction of Fe^{3+} with $^{\cdot}OH$:

$$Fe^{3+} + {}^{\cdot}OH \xrightarrow{k_2} [FeOH]^{3+} \qquad (2)$$

Whatever the species actually formed in reaction 1, we can designate it as the initiator (R_{init}) without changing the analysis of the reaction kinetics in-

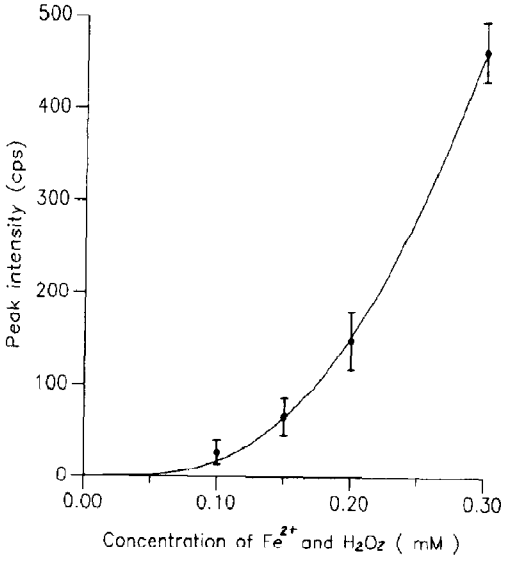

Fig. 4. Dependence of peak intensity of the chemiluminescence on the concentrations of Fe^{2+} and H_2O_2 under the conditions that both are of equal concentration and vary synchronously. Lipid peroxidation took place in 0.2 mM linoleic acid of 0.6% NaCl solution initiated by the reaction of Fe^{2+} with an equal concentration of H_2O_2 at 35° C. Bars indicate the standard deviation of the data. Curve was calculated by computer to fit the experimental data and described as $I = a\cdot[c]^4/(1 + b\cdot[c])^4$, where [c] is the concentration of Fe^{2+} or H_2O_2; constants a and b are 3.39×10^{17} M^{-1} cps and 1.87×10^3 M^{-1}, respectively.

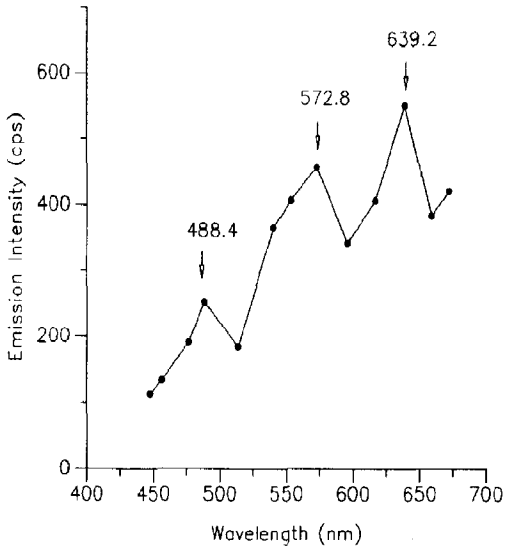

Fig. 5. Emission spectrum of chemiluminescence from the peroxidation of 0.2 mM linoleic acid initiated by reaction of 0.2 mM Fe^{2+} with 0.5 mM H_2O_2. Numbers above the arrows indicate the wavelengths of the interference filters. The spectrum has been corrected for the spectral response of the single-photon counter associated with the filters.

volved in the initiation, propagation and termination of lipid peroxidation.

The commonly accepted mechanism of free radical lipid peroxidation is outlined below (LH, fatty acid; LOOH, lipid peroxide; L$^{\cdot}$, lipid alkyl radical; LOO$^{\cdot}$, lipid peroxy radical) [12]:

$$\text{initiation: } LH + R_{init} \xrightarrow{k_3} L^{\cdot} + \text{reduced } R_{init} \quad (3)$$

$$\text{propagation: } L^{\cdot} + O_2 \xrightarrow{k_4} LOO^{\cdot} \quad (4)$$

$$LOO^{\cdot} + LH \xrightarrow{k_5} LOOH + L^{\cdot} \quad (5)$$

$$\text{termination: } LOO^{\cdot} + LOO^{\cdot} \xrightarrow{k_6} \text{nonradical product} + {}^1O_2 \quad (6)$$

$$L^{\cdot} + L^{\cdot} \xrightarrow{k_7} \text{nonradical product} \quad (7)$$

$$L^{\cdot} + LOO^{\cdot} \xrightarrow{k_8} \text{nonradical product} \quad (8)$$

In fact, the addition of oxygen to alkyl radical is a fast diffusion-controlled process ($k_4 = 10^9$–10^{10} $M^{-1}\,s^{-1}$) when the oxygen partial pressure of the reaction mixture is 100 mmHg or greater; only termination reactions involving the peroxy radical (i.e. reaction 6) need be considered [13].

Since the emitted photons in lipid peroxidation mainly come from the dimol reaction of $^1O_2$ as well as the excited carbonyls and both are the products of the reactions involving LOO$^{\cdot}$ [14], it is reasonable to assume that the peak intensity of photon emission should depend on the maximal concentration of LOO$^{\cdot}$ formed during the reactions and consequently on the maximal concentration of R_{init} from which the LOO$^{\cdot}$ is derived. The maximal concentration of the initiator can be estimated under three extreme conditions.

4.1. Under the condition: $[Fe^{2+}] \ll [H_2O_2]$

To a first approximation, the concentration of H_2O_2, $[H_2O_2]$, can be regarded as constant, the differential equation

$$\frac{d[Fe^{2+}]}{dt} = -k_1 \cdot [Fe^{2+}] \cdot [H_2O_2] \quad (9)$$

has the solution

$$[Fe^{2+}]_t = [Fe^{2+}]_0 \cdot e^{-k_1 \cdot [H_2O_2] \cdot t} \quad (10)$$

The concentration of the initiator is expressed as:

$$\frac{d[R_{init}]}{dt} = k_1' \cdot [Fe^{2+}] \cdot [H_2O_2] - k_3 \cdot [R_{init}][LH] \quad (11)$$

where k_1' is either the overall apparent rate constant of reaction 1 plus 2 or the same as k_1, depending on the nature of the actual oxidative species formed in the Fenton reaction. [LH], the concentration of linoleic acid, can be considered approximately to remain constant during peroxidation. At time t_c when $d[R_{init}]/dt = 0$, $[R_{init}]$ reaches its maximum:

$$[R_{init}]_{max} = \frac{k_1' \cdot [Fe^{2+}]_0 \cdot [H_2O_2]}{k_3 \cdot [LH]} e^{-k_1 \cdot [H_2O_2] \cdot t_c} \quad (12)$$

where $[Fe^{2+}]_0$ is the initial concentration of Fe^{2+}.

4.2. Under the condition: $[H_2O_2] \ll [Fe^{2+}]$

Approximately, the concentration of Fe^{2+}, $[Fe^{2+}]$, can be regarded as unchanged; the differential equation:

$$\frac{d[H_2O_2]}{dt} = -k_1 \cdot [Fe^{2+}] \cdot [H_2O_2] \quad (13)$$

can be solved as:

$$[H_2O_2]_t = [H_2O_2]_0 \cdot e^{-k_1 \cdot [Fe^{2+}] \cdot t} \quad (14)$$

Similarly to the treatment in section 4.1, the maximal concentration of the initiator can be estimated from eq. 15.

$$[R_{init}]_{max} = \frac{k_1' \cdot [Fe^{2+}] \cdot [H_2O_2]_0}{k_3 \cdot [LH]} e^{-k_1 \cdot [Fe^{2+}] \cdot t} \quad (15)$$

4.3. Under the condition: $[Fe^{2+}] = [H_2O_2]$

Eqs. 9 and 13 can be rewritten as

$$\frac{d[Fe^{2+}]}{dt} = -k_1 \cdot [Fe^{2+}]^2 \quad (16)$$

$$\frac{d[H_2O_2]}{dt} = -k_1 \cdot [H_2O_2]^2 \quad (17)$$

their solutions are:

$$[Fe^{2+}]_t = \frac{[Fe^{2+}]_0}{1 + k_1 \cdot [Fe^{2+}]_0 \cdot t} \quad (18)$$

$$[H_2O_2]_t = \frac{[H_2O_2]_0}{1 + k_1 \cdot [H_2O_2]_0 \cdot t} \quad (19)$$

Similarly to the previous treatment, let $d[R_{init}]/dt = 0$ in eq. 11; the maximal concentration of the initiator is estimated from eq. 20:

$$[R_{init}]_{max} = \left(\frac{k_1'}{k_3 \cdot [LH]}\right) \times \frac{[Fe^{2+}]_0 \cdot [H_2O_2]}{(1 + k_1 \cdot [Fe^{2+}]_0 \cdot t_c)(1 + k_1 \cdot [H_2O_2]_0 \cdot t_c)} \quad (20)$$

For $[Fe^{2+}]_0 = [H_2O_2]_0$, eq. 20 can also be rewritten as:

$$[R_{init}]_{max} = \frac{a_1 \cdot x^2}{(1 + b \cdot x)^2} \quad (21)$$

where x represents the initial concentration of Fe^{2+} or H_2O_2, the constant $a_1 = k_1/k_3 \cdot [LH]$, and $b = k_1' \cdot t_c$.

5. Discussion

The theoretical analysis shows that the maximal concentration of the initiator during lipid peroxidation is a linear function of the initial concentration of Fe^{2+} (or H_2O_2), provided that the second Fenton reagent is in great excess. The experimental data show that the peak intensity of the chemiluminescence is a quadratic function of the initial concentration of Fe^{2+} (or H_2O_2), provided that its initial concentration is much less than that of the other Fenton reagent. Correlating the theoretical analysis with the experimental observations, and assuming that the peak intensity of the chemiluminescence must be a function of the maximal level of the initiator which abstracts hydrogen from lipid and produces radicals and excited states, it may be reasonable to make a suggestion: the peak intensity of the chemiluminescence in the peroxidation of linoleic acid is, at least approximately, a quadratic function of the maximal concentration of the initiator. Thus, the peak intensity of the chemiluminescence, I, can be expressed as:

$$I = K \cdot [R_{init}]_{max}^2 \quad (22)$$

where K is a constant. In order to verify the validity of this suggestion, the dependence of the peak intensity on the initial concentration of Fe^{2+} and H_2O_2 was determined under the condition that both Fenton reagents have equal concentration. The theoretical treatment shows that the dependence of $[R_{init}]_{max}$ on the initial concentration of Fe^{2+} and H_2O_2 is described by eq. 20 or 21. Therefore, based on the suggestion expressed by eq. 22, we could expect the following function to relate the peak intensity of the chemilumines-

cence with the concentration of Fe^{2+} and H_2O_2:

$$I = \frac{a_1 \cdot K \cdot x^4}{(1 + b \cdot x)^4} \quad (23)$$

where x denotes the initial concentration of Fe^{2+} and H_2O_2. This function is precisely the same as that determined by experiment (see section 3.4 and fig. 4).

The emission spectrum of the chemiluminescence from the peroxidation of linoleic acid was determined with 13 interference filters which are available in the author's laboratory. Although the wavelength intervals between successive filters are not small enough and the wavelengths of the filters do not very well match the emission bands of dimol reactions of singlet oxygen, the emission spectrum measured still clearly shows the characteristic emission bands of singlet oxygen and is in good agreement with the emission spectrum reported for microsomal lipid peroxidation [15]. In particular, the two bands due to the transitions $2(^1\Delta g) \rightarrow 2(^3\Sigma g^-)$ are very prominent (see table 1). The emission band corresponding to the transition $(^1\Sigma g^+ + {}^1\Delta g) \rightarrow 2(^3\Sigma g^-)$ can also be detected, but appears to shift somewhat towards the red perhaps due to some systematic error in the measurements. In order to confirm the singlet oxygen origin of the luminescence, β-carotene, a good quencher of $^1O_2$, was added to the LA solution to observe its inhibition on the emission. It was found that 30 μM β-carotene can inhibit about 92% of the chemiluminescence. Therefore, we can conclude that the photon emission during the peroxidation of linoleic acid primarily comes from the dimol reaction of singlet oxygen; only a minor portion of the photons may derive from other source such as excited carbonyls.

Table 1

Some prominent emission bands from the dimol reaction of singlet oxygen

Numbers in parentheses indicate the vibronic components.

Reaction	Emission wavelength (nm)
$2\,(^1\Delta g)\,(0,0) \rightarrow 2\,(^3\Sigma g^-)$	635
$2\,(^1\Delta g)\,(1,0) \rightarrow 2\,(^3\Sigma g^-)$	578
$(^1\Sigma g^+ + {}^1\Delta g)\,(0,0) \rightarrow 2\,(^3\Sigma g^-)$	480

The results of the present study may imply that chemiluminescence is possibly a good index for reflecting more quantitatively the level and the effectiveness of the species which initiate the lipid peroxidation. The dependence of the peak emission intensity on the concentration of the initiator reported here may be potentially useful in the study of the mechanism of lipid peroxidation involving ferric iron or enzymes.

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